The Role of Gamma Interferon in Immune Resistance to Vaginal Infection by Herpes Simplex Virus Type 2 in Mice

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We investigated the role of interferon gamma (IFN-γ) in a mouse model of immunity to vaginal infection by herpes simplex virus type 2 (HSV-2). Within 8 h after immune mice were challenged intravaginally with HSV-2, IFN-γ concentrations in vaginal secretions reached levels that can be antiviral in vitro. This rapid synthesis of IFN-γ occurred in immune-challenged mice but not in nonimmune-challenged mice, indicating that it required memory T cells. Immunostaining and in situ hybridization revealed that the IFN-γ was synthesized by cells whose morphological appearance suggested that they were lymphocytes and macrophage-like cells in the mucosa. The presence of IFN-γ in vaginal secretions was correlated with upregulation of MHC class II antigens in the epithelium and with vigorous (30-fold) recruitment of T and B lymphocytes into the vagina. In vivo administration of anti-IFN-γ to immune mice 17 h before virus challenge blocked the subsequent appearance of IFN-γ in vaginal secretions, blocked upregulation of class II antigens, blocked adherence of T cells to endothelium and their recruitment into the vagina, and markedly reduced immunity against reinfection of the vaginal epithelium. © 1999 Academic Press

INTRODUCTION

An understanding of the immune mechanisms that protect the female genital tract against infections in animal models is essential for development of vaccines to protect women against sexually transmitted diseases (Parr and Parr, 1999). A mouse model of immunity against vaginal herpes simplex virus type 2 (HSV-2) infection has been described by McDermott and coworkers (1984) and modified by Parr et al. (1994). In this model, vaginal immunization with attenuated HSV-2 elicited immunity against a subsequent vaginal challenge with wild-type virus. The protective immunity in this model is quite strong (Parr and Parr, 1998a; Parr and Parr, 1998b). Twenty-four hours after immune mice were challenged in the vagina with wild-type virus, infection of the vaginal epithelium ranged from 0.0 to 2.5% of that measured in nonimmune mice, and at 72 h after vaginal challenge, no shed virus protein was detected in the vaginal lumen of immune mice whereas shed virus protein titers of 5000–6000 were present in nonimmune mice. No immune mice developed neurological illness, whereas nearly all nonimmune mice died 8–14 days after challenge. The dose of challenge virus used in these studies was 1000-fold higher than the minimum needed to cause lethal illness in nonimmune mice, thus vigorous immunity was needed to suppress the challenge infections so effectively. The data suggested that local immune mechanisms in the vaginal mucosa were important in this protection because replication of challenge virus in the vaginal epithelium was rapidly and effectively inhibited in the immune mice.

Antibody in vaginal secretions is an important component of immunity to vaginal HSV-2 infection (Parr and Parr, 1997). Affinity-purified IgG from vaginal secretions of adult immune mice, at its concentration in vivo in the vaginal mucus, effectively neutralized HSV-2, and purified IgG from serum of immune mice provided significant protection against epithelial infection after passive transfer to nonimmune mice. An involvement of cell-mediated immunity in the mouse vaginal HSV-2 model was first indicated by the observation that adoptive transfer of lymphocytes from the iliac lymph nodes of immune mice protected naive mice against neurological illness after vaginal challenge with wild-type virus (McDermott et al., 1987). We further investigated the role of T cells in vaginal immunity by in vivo depletion of these cells in immune mice 1 week before vaginal challenge (Parr and Parr, 1998b). Acute depletion of T cells for this short period had no effect on antibody titers in vaginal secretions at the time of challenge. The results showed that immune mice depleted of CD4+ and CD8+ cells, Thy-1+ cells, or CD8+ cells alone had greater viral infection of the vaginal epithelium 24 h after challenge than did nondepleted immune mice. The T cells of immunized mice thus acted rapidly to protect the vaginal epithelium against infection by the challenge virus. Similar results have been reported by Milligan et al. (1998).

The protective effect of T cells against numerous intracellular pathogens is partly due to their secretion of

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gamma interferon (IFN-γ) after activation by antigen (Buchmeier and Schreiber, 1985; Li et al., 1987; Suzuki et al., 1988; Squires et al., 1989; Zhong et al., 1989; Ramsay et al., 1993; Cantin et al., 1995). In the mouse vaginal HSV-2 model, IFN-γ is probably secreted in the vaginal mucosa of immune mice after virus challenge because MHC class II antigens in vaginal epithelial cells were upregulated (Parr et al., 1994), and it is well known that IFN-γ is a potent inducer of class II antigens (Steeg et al., 1982; Poher et al., 1983; Basham et al., 1985; Bland, 1988). Moreover, Milligan and co-workers (Milligan and Bernstein, 1997; Milligan et al., 1998) have shown that IFN-γ is involved in clearance of HSV-2 from the vagina of non-immune mice and in resistance to reinfection in immune mice. In the present study, we have further investigated the role of IFN-γ in immunity to vaginal HSV-2 infection. In particular, we measured the concentration of IFN-γ in the vaginal lumen of immune mice after challenge as an indication whether it may have antiviral activity in the adjacent epithelial layer and localized it in the vaginal mucosa by immunostaining and in situ hybridization. We also determined the time course of IFN-γ secretion after virus challenge in both immune and nonimmune mice and correlated that secretion to the expression of MHC class II antigens in the epithelium and to the numbers of T and B lymphocytes in the mucosa. Finally, we evaluated in vivo neutralization of IFN-γ by administration of monoclonal antibody and the effects of such neutralization on upregulation of epithelial MHC class II antigens, on recruitment of lymphocytes to the vagina, and on immunity to re-infection of the epithelium by HSV-2. The results of these studies demonstrate an important role for memory T cells and IFN-γ in the early stages of resistance to challenge virus infection of the vaginal epithelium.

RESULTS

IFN-γ in vaginal secretions

The concentrations of IFN-γ in extracts of vaginal mucus from individual mice were measured by chemiluminescence ELISA (Table 1). IFN-γ was not detectable in vaginal secretions from nonimmune or immune mice, but it was readily detected 20 h after immune mice were challenged in the vagina with wild-type HSV-2. The ELISA reaction for IFN-γ in the latter vaginal samples was inhibited 90% by addition of rat monoclonal anti-mouse IFN-γ at 100 ng/ml, indicating specificity for IFN-γ. Based on a mean concentration of 150 pg/ml in the extracts of vaginal mucus from immune-challenged mice and a 15-fold dilution during extraction, the concentration of IFN-γ in situ in the vaginal secretions of these mice was ~2.0 ng/ml.

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonimmune</td>
<td>0.44 ± 0.64</td>
</tr>
<tr>
<td>Immune†</td>
<td>−0.88 ± 0.55</td>
</tr>
<tr>
<td>Immune/challenged‡</td>
<td>150 ± 14</td>
</tr>
</tbody>
</table>

† Ten mice per group.
‡ Not significantly different from 0.0, *P = 0.51, two-tailed Student’s *t* test.
§ Six weeks after vaginal immunization with attenuated HSV-2 at 1.5 × 10⁵ PFU/ml.
¶ Not significantly different from 0.0, *P = 0.15, two-tailed Student’s *t* test.
|| Twenty hours after immune mice were challenged in the vagina with wild-type HSV-2 at 3.5 × 10⁵ PFU/ml.
|                          |               |

IFN-γ in vaginal mucosa

The source (s) of IFN-γ in vaginal secretions was investigated by immunostaining and in situ hybridization. Immunostaining of IFN-γ was observed in clusters of irregularly shaped, macrophage-like cells located just under the vaginal epithelium at 24 h after immune mice were challenged (Figs. 1a and 1b). Little or no staining was detectable in macrophage-like cells in the remainder of the stroma. IFN-γ was also observed in smaller cells that appeared to be lymphocytes within the clusters of macrophage-like cells and in similar cells scattered throughout the stroma. No staining was present in these sections when irrelevant rabbit antisera were substituted for rabbit anti-mouse IFN-γ serum (Fig. 1c), and staining was markedly inhibited when anti-IFN-γ serum was neutralized with recombinant IFN-γ before application to the sections. Also no staining of IFN-γ was detectable in sections from immune-nonchallenged mice or in sections from nonimmune mice at 24 h after challenge.

Using high-stringency conditions of in situ hybridization, IFN-γ mRNA was observed in clusters of macrophage-like cells located just under the vaginal epithelium (Fig. 2a) and in similar cells scattered throughout the stroma in immune mice at 24 h after challenge. IFN-γ mRNA was also observed in lymphocyte-like cells that were arranged in clusters or scattered throughout the stroma and in similar cells in the basal region of the epithelium (Fig. 2a). Little or no nonspecific staining was observed when the sense probe was used on these sections (Fig. 2b) or when antisense probe was used on sections from immune-nonchallenged mice or nonimmune-challenged mice.
Time course of IFN-γ secretion

The concentration of IFN-γ in vaginal secretions of immune mice increased rapidly after vaginal challenge with wild-type virus, reaching maximum levels within 8 h and then declining slowly to a low level at 96 h after challenge (Table 2). The IFN-γ concentration in the superficial vaginal mucosa thus exceeded 1.0 ng/ml within 8 h after challenge and remained above that level for ~24 h. Uptake of MHC class II antigens in vaginal epithelial cells lagged behind IFN-γ concentrations, becoming detectable at 16 h and reaching maximum at 24–32 h after challenge (Fig. 3a). The numbers of lymphocytes in the vaginal mucosa also increased rapidly after vaginal challenge, being significantly increased at 8 h and reaching near maximum levels by 24 h after challenge (Figs. 4a, 4c, and 4e). In nonimmune mice, IFN-γ was not detectable in vaginal secretions until 48 h after challenge (Table 3). At this time T and B cell numbers were not increased in the vagina, and MHC class II antigens were just beginning to appear in vaginal epithelial cells. The contrasting results in immune and nonimmune mice strongly suggest that memory T cells in the vagina of immune mice responded to the challenge virus antigen by rapid secretion of IFN-γ and that some aspect of the memory T cell response also upregulated MHC class II antigens in the epithelium and caused vigorous recruitment of T and B lymphocytes into the vagina.

Neutralization of IFN-γ in vivo

Administration of ≥0.3 ml of anti-IFN-γ ascites to immune mice 17 h before vaginal challenge effectively neutralized or eliminated IFN-γ in vaginal secretions 24 h after challenge and blocked upregulation of MHC class II antigens in the vaginal epithelium (Table 4; Fig. 3b). Unexpectedly, the in vivo neutralization of IFN-γ also completely blocked recruitment of T cells to the vagina and diminished recruitment of B cells while having no apparent effect on neutrophils (Figs. 4b, 4d, and 4f). Local IFN-γ secretion by memory T cells in the vagina after antigen challenge was thus required for recruitment of additional lymphocytes to the vagina. The effect of IFN-γ on recruitment of lymphocytes to the vagina involved the vascular endothelium because many lymphocytes were adherent to the endothelium in immune-challenged mice without ascites treatment (Fig. 5), but negligible adherent T cells and few B cells were present in small veins of immune-challenged mice treated with either 0.3 or 1.0 ml of ascites (Figs. 4b, 4d, and 4f).

### Table 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IFN-γ (pg/ml)</th>
<th>MHC class II</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>0</td>
<td>0.7 ± 0.7</td>
<td>0.0 ± 0.0</td>
<td>0.36 ± 0.07</td>
</tr>
<tr>
<td>8</td>
<td>191 ± 15.0</td>
<td>0.0 ± 0.0</td>
<td>5.61 ± 1.02</td>
</tr>
<tr>
<td>16</td>
<td>139.0 ± 15.0</td>
<td>1.3 ± 0.2</td>
<td>8.9 ± 1.7</td>
</tr>
<tr>
<td>24</td>
<td>81.0 ± 8.0</td>
<td>2.7 ± 0.2</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>32</td>
<td>81.0 ± 12.0</td>
<td>3.0 ± 0.0</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>48</td>
<td>220 ± 6.5</td>
<td>1.6 ± 0.2</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>96</td>
<td>7.6 ± 2.0</td>
<td>0.4 ± 0.1</td>
<td>3.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Time after vaginal challenge with wild-type HSV-2. A total of 57 mice were used with 5–10 mice at each time point.
* IFN-γ concentration in 200-μl extracts of vaginal mucus (mean ± standard error of mean).
* Staining of vaginal epithelial cells on a scale of 0–3. One section from each of two blocks from each mouse were examined.
* Numbers of lymphocytes per high power microscopic field of vaginal stroma (mean ± standard error of mean). Five fields from each of two histological sections from two blocks from each mouse were counted. The numbers of CD4+, CD8+, and B220+ lymphocytes at 8 h after challenge were significantly larger than the numbers present at 0 h (P = 0.0017, P = 0.016, and P = 0.0018, respectively, one-tailed t tests).
Effects of in vivo neutralization of IFN-γ on immunity to epithelial infection

As expected from the results in Table 4, administration of 0.5 ml of anti-IFN-γ ascites to immune mice 17 h before challenge neutralized or eliminated IFN-γ in vaginal secretions after challenge and blocked upregulation of MHC class II antigens in the vaginal epithelium (Table 5). Similarly, in vivo neutralization of IFN-γ again blocked adherence of T cells to the vascular endothelium, blocked recruitment of both CD4+ and CD8+ T cells into the vaginal mucosa, and reduced recruitment of B cells. Ascites treatment had no significant effect on IgG anti-HSV-2 titers in vaginal secretions at 24 h after challenge but caused a small increase in antibody titers at 52 h after challenge. Virus protein shedding from the vaginal epithelium of immune mice after challenge was markedly increased by in vivo neutralization of IFN-γ as evidenced by increased concentrations of shed virus proteins in the vaginal secretions of ascites-treated mice at both 24 and 52 h after challenge. However, all immunized mice remained immune to lethal neurological disease. Thus antibody and possibly other immune mechanisms that remained effective after in vivo neutralization of IFN-γ were sufficient to prevent neurological disease. This illustrates clearly that if the intent of an experiment is to evaluate the immune mechanisms that act at the mucosal surface to prevent challenge infection of the vaginal epithelium, then a direct measure of epithelial infection as employed here is more informative than neurological disease.

DISCUSSION

IFN-γ was detected in the vaginal secretions within 8 h after immune mice were challenged in the vagina with HSV-2. At 24 h after challenge, the cytokine was localized in the vaginal mucosa by in situ hybridization and immunostaining in cells having the morphology of lymphocytes and macrophages; further studies are needed to clarify the identity of these cells. After secretion in the mucosa, the cytokine probably passed into the vaginal lumen by transudation and accumulated in the mucus that is secreted by the vaginal epithelium. Because the volume of the mucus secretion in the vaginal lumen is known (Parr et al., 1998), we were able to calculate the concentration of IFN-γ in situ in the vaginal lumen. This concentration exceeded 1.0 ng/ml (10 IU/ml) between 8 and 32 h after challenge, and the concentration in the mucosa was presumably higher than in the lumen. In a similar study, Milligan and colleagues (1998) reported IFN-γ concentrations in the vaginal lumen that were higher than those reported here. IFN-γ concentrations of ~10 IU/ml have been found to inhibit replication of several kinds of viruses in diverse target cells in vitro (Babiuk and Rouse, 1976; Koyanagi et al., 1988; Mestan et al., 1988; Kornbluth et al., 1989; Croen, 1993; Karupiah et al., 1993). While many possibilities exist for synergism or antagonism between IFN-γ and other cytokines in vivo, the data are consistent with a suggestion that IFN-γ might directly inhibit the replication of sexually transmitted intracellular pathogens such as HSV-2, human papillomaviruses, human immunodeficiency virus–1, and Chlamydia trachomatis in the cervix or vagina of appropriately immunized humans. We observed that virus shedding in the vagina of immune-challenged mice was substantially increased when IFN-γ was neutralized in vivo with monoclonal antibody, but it is important to recognize that this could be due to the inhibition of T cell recruitment to the vagina rather than to inhibition of direct antiviral effects. It has been suggested that direct antiviral effects of IFN-γ in vivo may be less important than immunoregulatory effects (Nathan, 1992; Farrar and Schreiber, 1993), but this is based almost entirely on the
FIG. 4. Fluorescence micrographs showing immunolabeling of CD4+ T cells (a, arrows), CD8+ T cells (c, arrows), and B220+ B cells (e, arrows) in the vaginal stroma of immune mice 24 h after ivag challenge with wild-type virus. Little or no labeling of CD4+ (b), CD8+ (d), and B220+ cells (f) was observed in the vaginas of immune-challenged mice that were treated with anti-IFN-γ 17 h before challenge. Nonspecific staining is due to fluorescence of granulocytes. E, epithelium. Arrowheads, blood vessels. Magnifications: all X350.

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clearance of primary infections rather than on immunity against challenge infections.

Memory T cells are distinguished from naive T cells mainly by their more rapid and vigorous response to antigen, including their cytokine secretory response (Murali-Krishna et al., 1998). The present demonstration that IFN-γ was rapidly secreted (<8 h) in the vagina in response to virus challenge in immune mice but not in nonimmune mice thus provides cogent in vivo evidence that functional, virus-specific memory T cells were present in the vagina at the time of challenge. Our results in vivo are analogous to early in vitro studies showing that T lymphocytes from immune humans (Valle et al., 1975) and cows (Babiuk and Rouse, 1976), when cultured with macrophages and antigen, secreted IFN-γ into the medium in as little as 8 h, whereas T cells from nonimmune individuals did not produce IFN-γ. This interpretation of the kinetic data is consistent with the theory that memory T cells preferentially recirculate from blood to lymph via nonlymphoid tissues, whereas naive T cells preferentially recirculate via lymph nodes (Mackay, 1991).

We have shown that many if not most of the T cells that continuously traffic from the superficial vaginal mucosa to the iliac lymph nodes of both immune and nonimmune mice bear the memory T cell marker, CD44 (King et al., 1998). Further evidence that memory T cells in the vagina belong to the recirculating pool comes from the observation that vaginal challenge elicited rapid upregulation of MHC class II antigens and recruitment of lymphocytes to the vagina in parenterally immunized mice (Parr and Parr, 1998a) and rapid secretion of IFN-γ in nasally immunized mice (Parr and Parr, unpublished observations). Also, virus–specific effector T cells have been demonstrated in the female reproductive tract (Lohman et al., 1995; Milligan and Bernstein, 1995). Our present results complement a report that the IFN-γ response to HSV-2 challenge in this immune mouse model was abrogated by in vivo depletion of T cells with anti-Thy-1 antibody (Milligan et al., 1998). Because virus shedding in the vagina of immune mice after challenge was substantially

### TABLE 3

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IFN-γ (pg/ml)</th>
<th>MHC class II</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>0</td>
<td>5.4 ± 11.4</td>
<td>0.0 ± 0.0</td>
<td>0.32 ± 0.22</td>
</tr>
<tr>
<td>24</td>
<td>1.3 ± 11.5</td>
<td>0.0 ± 0.0</td>
<td>0.32 ± 0.15</td>
</tr>
<tr>
<td>32</td>
<td>&lt;5.5 ± 11.7</td>
<td>0.0 ± 0.0</td>
<td>0.02 ± 0.06</td>
</tr>
<tr>
<td>48</td>
<td>250 ± 34</td>
<td>0.11 ± 0.05</td>
<td>0.50 ± 0.20</td>
</tr>
<tr>
<td>96</td>
<td>290 ± 60</td>
<td>0.88 ± 0.25</td>
<td>2.24 ± 1.26</td>
</tr>
</tbody>
</table>

* Time after vaginal challenge with wild-type HSV-2. A total of 40 mice were used with 5–10 mice at each time point.
* IFN-γ concentration in 200 μl extracts of vaginal mucus (mean ± standard error of mean). Values at 0, 24, and 32 h are not significantly different from 0.0 (two-tailed t tests).
* Staining of vaginal epithelial cells on a scale of 0–3. One section from each of two blocks from each mouse was examined.

### TABLE 4

<table>
<thead>
<tr>
<th>Anti-IFN-γ (ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>MHC class II</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>0.0</td>
<td>110 ± 18</td>
<td>3.0 ± 0.0</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>0.1</td>
<td>9.9 ± 2.0</td>
<td>0.004 ± 0.003</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>0.3</td>
<td>3.8 ± 1.4</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>1.0</td>
<td>−0.7 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* Anti-IFN-γ ascites was injected ip into immune mice 17 h before vaginal challenge with HSV-2. The monoclonal antibody doses were 0.0, 0.2, 0.6, and 2.0 mg of rat IgG anti-mouse IFN-γ, respectively. A total of 37 mice were used with 9–10 mice in each group.
* IFN-γ concentration in 200 μl extracts of vaginal mucus 24 h after challenge with wild-type virus (mean ± standard error of mean).
* Staining of vaginal epithelial cells on a scale of 0–3 (mean ± standard error of mean). One section from each of two blocks from each mouse was examined.
* Numbers of lymphocytes per high power microscopic field of vaginal stroma (mean ± standard error of mean). Five fields from each of two sections from two blocks from each mouse were counted.
increased when IFN-γ was neutralized in vivo, it is apparent that the virus-specific memory T cells that recirculate through the vagina play a key early role in mucosal immunity to vaginal HSV–2 infection even if their only function is that of rapid IFN–γ secretion in response to antigen challenge.

The early involvement of memory T cells in immunity against vaginal reinfection by HSV–2 emphasizes the importance of factors that may influence the numbers of memory lymphocytes in the vagina after immunization but before challenge. A particularly important consideration is whether the site of immunization affects the numbers of either sessile or recirculating memory T cells in the vagina. Studies of the intestine and skin have suggested that memory lymphocytes that were activated in those locations later recirculated preferentially through those tissues (Mackay et al., 1992; Picker and Butcher, 1992; Mackay, 1994). This suggests that local immunization in the vagina might result in greater numbers of memory lymphocytes in the vagina than would be generated by an otherwise equivalent immunization at a parenteral site. In a recent comparison of vaginal and parenteral immunization with attenuated HSV–2, we found only a modest difference in the numbers of T and B lymphocytes in the vagina of the immune mice before virus challenge and little difference in recruitment of lymphocytes to the vagina after challenge (Parr and Parr, 1998a). Vaginal lymphocyte numbers were approximately twofold higher in vaginally immunized mice than in the parenteral groups, but serum antibody titers were also twofold higher in the vaginally immunized mice, suggesting a more efficient immunization at this site and possibly a greater proliferation of lymphocytes. Another possibility is that lymphocyte homing and recruitment in the vagina may be influenced by steroid hormones. All mice in the present study were treated with a progestin, and comparisons with estrogen treatment or other treatments remain to be explored.

Previously we observed that both T and B lymphocytes were recruited to the vagina within 24 h after virus challenge in immune mice but not in nonimmune mice; that is, the kinetics of lymphocyte recruitment indicated that it involved a memory response (Parr and Parr, 1998a). We confirmed and extended that observation in the present study, finding that lymphocyte recruitment in immune mice was already well underway within 8 h after vaginal challenge and that the recruitment required IFN-γ. Direct injection of IFN–γ into the skin of nonimmunized rats (Issekutz et al., 1988) and baboons (Munro et al., 1989) caused migration of leukocytes into the tissue, and administration of anti–IFN–γ to mice during primary infection with influenza virus inhibited leukocyte infiltration into the lungs (Baumgarth and Kelso, 1996). Similarly, administration of anti–IFN–γ in vivo caused a 50–90% inhibition of leukocyte recruitment into a site of delayed–type hypersensitivity (Issekutz et al., 1988). In contrast, recruitment of T cells to the lungs of immune mice challenged with influenza virus was reported to be not significantly different in normal and IFN–γ–knockout mice (Bot et al., 1998). We are not aware of any previous studies showing that IFN–γ mediates rapid recruitment of large numbers of T and B lymphocytes to a site of antigen challenge in immunized animals, and such recruitment is not currently recognized as a function of...
IFN-γ (Dijkmans and Billiau, 1988; Nathan, 1992; Farrar and Schreiber, 1993; Springer, 1994; Billiau, 1996). Nevertheless, our data clearly indicate that the IFN-γ secreted by memory T cells in the vagina of immune mice after virus challenge was responsible for rapid recruitment (>8 h) of large numbers (~30-fold increase) of additional T and B lymphocytes to the vagina.

Large numbers of T and B lymphocytes were present in the small veins in the vagina of immune mice after challenge, but T cells were virtually absent from such vessels if the mice were pretreated with anti–IFN-γ. Thus recruitment of T lymphocytes into the vagina by IFN-γ appeared to involve the induction of adhesion factors to the vascular endothelium. The adhesion molecules ICAM-1 and VCAM-1 and their respective lymphocyte integrins LFA-1 and VLA-4 may play a role in this experimental system because they mediate T lymphocyte recruitment into tissues (Dustin and Springer, 1988; Baron et al., 1993; Perry et al., 1998); the adhesion molecules may be upregulated on vascular endothelial cells by IFN-γ (Dustin et al., 1986; Yu et al., 1985; Ruszczak et al., 1990; Wellicome et al., 1990; Bevilacqua, 1993; Lechleitner et al., 1998); and in vivo administration of monoclonal antibodies to LFA-1 and VLA-4 blocked the IFN-γ-mediated recruitment of lymphocytes into the site of a delayed-type hypersensitivity reaction (Chisholm et al., 1993; Isssekutz, 1993; Scheynius et al., 1993; Springer, 1994).

### MATERIALS AND METHODS

#### Animals and virus

Female BALB/c mice were purchased from Harlan/Sprague–Dawley, Indianapolis, IN, and were 10–20 weeks old when used. They were housed in compliance with all institutional and federal animal welfare requirements, and all experimental procedures were approved by the institutional Animal Care and Use Committee. Wild-type TK+ HSV-2 and attenuated ΔTK+HSV-2, a strain that contains a partial deletion of the thymidine kinase gene; HSV-2-infected Vero cell lysates; and uninfected Vero cell lysates were generously provided by Dr. Mark McDermott, McMaster University, Hamilton, Canada (McDermott et al., 1984, 1987).

#### Vaginal immunization and challenge

Mice were immunized by pretreatment with 2.0 mg of Depo-Provera (DP) (Upjohn Co., Kalamazoo, MI) in phosphate-buffered saline (PBS) subcutaneously, followed 6
days later by intravaginal (ivag) inoculation of 20 \( \mu l \) of attenuated HSV-2 at \( 1.5 \times 10^8 \) PFU/ml. Immune and age-matched nonimmune mice were challenged by pre-treatment with DP as above, followed 6 days later by ivag inoculation of 10 \( \mu l \) of wild-type HSV-2 at \( 3.5 \times 10^6 \) PFU/ml.

**Vaginal secretion**

The vaginal lumen of progestin-treated mice is filled with mucus that is 97% water and has a volume of 10–15 \( \mu l \) (Parr et al., 1998). The vaginal mucus was collected once from each mouse by pipetting 50 \( \mu l \) of Tris-buffered saline (TBS) at pH 7.5 in and out of the vagina until the mucus was recovered. The vagina was immediately washed with 50 \( \mu l \) of TBS again to ensure effective recovery of the vaginal secretions. Afterward the two washes were combined and frozen at \(-20^\circ\)C until needed. The mucus was extracted by rotating thawed samples at 15 rpm for 2 h at \( 4^\circ\)C, followed by centrifugation to pellet the mucus. The supernatant was recovered and replaced by 120 \( \mu l \) of fresh TBS, followed by rotation for 2 h at \( 4^\circ\)C. The two mucus extracts were combined, brought to 0.05% Tween 20, adjusted to 200 \( \mu l \) (some TBS is absorbed by the mucus during extraction), then diluted with 200 \( \mu l \) of the casein-containing diluent that was used for all ELISA measurements (see below). The resulting 400–\( \mu l \) samples from each mouse were used for all ELISA measurements, and measured concentrations and titers were doubled so that all results refer to concentrations in the 200–\( \mu l \) extracts of vaginal mucus. Because mucus volumes were 10–15 \( \mu l \), concentrations \textit{in situ} in the vagina were \(<15\text{-fold higher than those measured in the extracts}.

**Measurement of IFN–\( \gamma \) by ELISA**

Capture antibody (monoclonal rat anti-mouse IFN–\( \gamma \), clone R4–6A2 (Access Biomedical, San Diego, CA) at 6 \( \mu g/ml \) was bound to high-binding (Costar, Cambridge, MA) microtiter plate wells overnight in 0.10 M carbonate buffer at pH 9.5. After being washed in PBS–0.05% Tween 20, plate wells were blocked for 5 h with 0.20% casein (Tropix Inc., Bedford, MA) in PBS–0.10% Tween 20 at pH 6.8. Samples (100 \( \mu l \)), diluted as required in PBS at pH 7.5 containing 0.20% casein and 0.05% Tween 20, were then placed in the wells and incubated overnight at \( 4^\circ\)C. After washing in PBS–Tween 20, bound IFN–\( \gamma \) was detected with rabbit anti-mouse recombinant IFN–\( \gamma \) (Biosource International, Inc., Camarillo, CA) in dilution medium for 2 h, followed by washing in PBS–Tween 20 and incubation for 2 h in alkaline phosphatase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) in dilution medium. After being washed in PBS–Tween 20, plate wells were incubated with 100 \( \mu l \) of chemiluminescence substrate (CSPD with sapphire, Tropix Inc., Bedford, MA), followed by measurement in a Microlite (R) ML2250 luminometer (Dynatech Laboratories, Alexandria, VA). Recombinant dimeric mouse IFN–\( \gamma \) (PharMingen, San Diego, CA, 10 IU/ng) was used as the standard and could be detected at 2.0 pg/ml and higher (sample mean larger than blank mean plus 3 standard deviations). The IFN–\( \gamma \) in vaginal secretions was stable during storage at \(-20^\circ\)C, but its concentration declined 10–20% per day in extracts of vaginal mucus that were stored at \( 4^\circ\)C. Therefore IFN–\( \gamma \) was always measured on the day the vaginal mucus samples were thawed and extracted.

**Measurement of IgG anti-HSV-2 by ELISA**

Microtiter plate wells (high-binding white plates, Costar) were filled with 100 \( \mu l \) of UV-inactivated lysate of HSV-2-infected Vero cells in carbonate buffer at pH 9.5, covered with sealing film, centrifuged at 2700 rpm for 2 h in a Beckman GS-6R centrifuge, and incubated overnight at \( 4^\circ\)C. On the next day, plate wells were washed with PBS–0.05% Tween 20 and blocked 5 h with 0.20% casein (Tropix Inc.) in PBS–0.10% Tween 20 at pH 6.8. Serial 2.5-fold dilutions of samples in PBS containing 0.20% casein and 0.05% Tween 20 at pH 7.5 were then placed in the wells and incubated overnight at \( 4^\circ\)C. After washing in PBS–Tween 20, the wells received alkaline phosphatase-conjugated goat anti-mouse IgG (Fc) (Jackson Immunoresearch Labs Inc., West Grove, PA) for 2 h, followed by washing and introduction of substrate (CSPD with sapphire, Tropix). Luminescence was measured in a Microlite (R) ML 2250 luminometer (Dynatech Laboratories, Alexandria, VA). The sample antibody titer was defined as the reciprocal of the sample dilution at which the luminescence declined to the mean value of nonimmune samples at the same dilution plus 3 standard deviations. \( \log_{2.5} \) geometric mean titers and their standard errors were measured for each group and used to determine the statistical significance of differences between groups. Control measurements demonstrated that all ELISA reactions were at the same background level when immune and nonimmune samples were incubated on lysates of uninfected Vero cells and when nonimmune samples were incubated on infected Vero cells.

**Measurement of shed virus protein by ELISA**

Shed virus protein was measured by ELISA in extracts of vaginal mucus that was collected at 24 and 52 h after vaginal challenge with wild-type virus. The method was similar to that recently described by Franco and Greenberg (1995) and Parr and Parr (1997). The resulting shed virus protein titers are closely correlated with the percentage of the vaginal epithelium that is infected by the virus, indicating that the method measures virus protein that is shed into the vaginal lumen from infected epithe-
Immunolabeling of IFN-γ was used with the following modifications. The capture antibody was rabbit IgG anti-HSV-1 and 2 (Dako Corp., Carpinteria, CA) at 10 μg/ml. For detection of captured virus proteins, the rabbit IgG anti-HSV was conjugated to biotin-X-NHS (Calbiochem Corp., LaJolla, CA), and the biotinylated antibody was detected with alkaline phosphatase-streptavidin (Pierce Chemical Co., Rockford, IL). Serial 2.5-fold dilutions of the vaginal samples were measured. Titer was defined as the reciprocal of the dilution at which the luminescence declined to 3 standard deviations above the mean background luminescence in wells containing samples from immune-nonchallenged mice at the same dilution. Log_{2.5} geometric mean titters and their standard errors were measured for each group and used to determine the statistical significance of differences between groups, and geometric mean titters are also presented because they indicate more clearly the relative concentrations of shed virus proteins in the groups.

### Tissues

For immunolabeling, vaginae from 10 nonimmune-challenged, 10 immune-challenged, and 10 immune-non-challenged mice were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4°C, 2 h), washed with PBS containing 10% sucrose (4°C, 2 h), embedded in O.C.T. (Tissue-Tek, Miles Scientific, Naperville, IL), frozen in isopentane cooled with liquid nitrogen, and stored at −70°C until needed. Cryostat sections (6 μm) were mounted on silanized slides, air dried, and stored with desiccant at −20°C until needed. Sections from both ends of each vagina were studied. For *in situ* hybridization, 30 vaginae from the three mouse groups mentioned above were fixed in 4% paraformaldehyde (4°C, overnight) and embedded in paraffin. Sections from both ends of each vagina were studied.

### Immunolabeling of IFN-γ

Tissue sections were blocked in 2% normal goat serum (30 min), incubated in rabbit anti-mouse IFN-γ (60 min, 37°C, Biosource International Inc.), washed in PBS (10 min), treated with 0.5% hydrogen peroxide in methanol, washed in PBS, incubated in biotinylated goat anti-rabbit IgG (1/150, 20 min, Vector Labs Inc., Burlingame, CA) followed by streptavidin peroxidase (10 min, Zymed Labs. Inc.), and exposed to substrate (AEC kit, Zymed Labs.). Tissue sections were then counterstained with Gill's haematoxylin and mounted in Gelmount (Biomeda Corp., Foster City, CA). Specificity of labeling was indicated by the absence of staining when irrelevant rabbit IgG antibodies were substituted for the primary antibody by marked inhibition of labeling when primary antibody was mixed with recombinant IFN-γ (PharMingen) at 10 μg/ml prior to labeling and by absence of labeling in sections from immune-nonchallenged mice.

### IFN-γ *in situ* hybridization

A cDNA probe for interferon-γ was generously supplied by Genentech, Inc. (San Francisco, CA). The plasmid (pms10) contained the cDNA inserted in the *Pst*I site of pBR322. The IFN-γ cDNA served as a template for polymerase-directed synthesis of digoxigenin-labeled antisense and sense cRNA (Genius Nucleic Acid Detection Kit, Boehringer-Mannheim Corp., Indianapolis, IN). For *in situ* hybridization, tissue sections were deparaffinized, rehydrated in graded ethyl alcohols, treated with 1 μg/ml proteinase-K for 30 min at 37°C, and washed for 10 min in PBS, pH 7.2, containing 5 mM MgCl₂. The sections were prehybridized in 50% formamide/double-strength standard sodium citrate buffer (SSC; 20-strength SSC contains 3 M NaCl and 0.3 M sodium citrate), pH 7.0, at 66°C. Sections were incubated overnight at 47°C in a humidified chamber with a hybridization cocktail containing 50% formamide, 10% dextran sulfate, 250 μg trRNA/ml, and 100 ng/ml of either antisense RNA IFN-γ probe or the sense version of the same probe for control sections. After hybridization, tissue sections were washed extensively, concluding with 0.1× SSC at 47°C for 30 min. Hybridization was detected by incubating the sections with biotinylated anti-digoxigenin antibody (Sigma Chemical Co., St. Louis, MO) followed by treatment with avidin conjugated to alkaline phosphatase (Sigma). Incubation in a substrate solution containing levamisole, nitroblue-tetrazolium, and 5-bromo, 4-chloro, 3-indolyl phosphate (Promega, Madison, WI) yielded a brown reaction product at the site of hybridization between IFN-γ mRNA and probe. Tissue sections were counterstained with nuclear fast red.

### Quantitation of lymphocytes

Cryostat sections were post-fixed 10 min in acetone, blocked in 2% in 2% goat serum, and incubated 1 h in one of the following primary antibodies: rat anti-mouse CD4 or CD8 (Becton Dickinson, Mountain View, CA) or rat anti-mouse B220 (ATCC, Rockville, MD). The secondary antibody was FITC-donkey anti-rat IgG (Chemicon International, El Segundo, CA). Specificity of labeling was indicated by the absence of staining when normal rat IgG was substituted for the primary antibodies. Lymphocytes (CD4+, CD8+, B220+) were counted in five randomly selected high power fields from each of two separate regions of vagina. The stained cells were counted in captured images using an image analysis system. A high-resolution RGB color camera with integration (AlC-O-VI 470, Hyper HAD CCD; Optronics Engineering, Goleta, CA) was attached to the fluorescence microscope. Captured images were analyzed using a Macintosh computer (Quadra 840 AV) equipped with the LG-3 frame.
graber (Scion Corp. Frederick, MD) and using NIH Image 1.55 (Wayne Rasband, National Institutes of Health).

Quantitation of MHC class II antigen staining in vaginal epithelium

Sections of vagina were stained as described above for lymphocytes, using rat anti-mouse Ia (Boehringer-Mannheim) as the primary antibody. The intensity of staining in the vaginal epithelium was evaluated in two coded sections from each mouse and recorded as none (0), weak (1), moderate (2), or bright (3), along with the approximate proportion of epithelium with each kind of staining. All staining was evaluated in captured images.

In vivo depletion of IFN-γ

Hybridoma cell line R4–6A2 (rat anti-murine IFN-γ) was purchased from ATCC. Ascites fluid containing the monoclonal antibody was produced by TSD BioServices (Germantown, NY) by inoculating cultured hybridoma cells into pristane-primed nude mice. The ascites fluids was purchased from ATCC. Ascites fluid containing the monoclonal antibody was produced by TSD BioServices (Germantown, NY) by inoculating cultured hybridoma cells into pristane-primed nude mice. The ascites fluids was purchased from ATCC. Ascites fluid containing the monoclonal antibody was produced by TSD BioServices (Germantown, NY) by inoculating cultured hybridoma cells into pristane-primed nude mice. The ascites fluids

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Illness scores

Illness was indicated by ruffled fur, arched backs, feeble movements, paralysis of one or both hindlimbs, and by a swollen red vulva. An illness score of 3.0 was assigned to mice that died or became so ill that euthanasia was desirable by 9 days after challenge with wild-type virus. Mice that died or required euthanasia from 10 to 14 days after challenge were scored 2.0. Mice that developed some sign of illness but survived beyond 14 days were scored 1.0. Mice that never showed signs of illness were scored 0.0.

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